Journal of Chemical Ecology, Vol. 31, No. 7, July 2005 (©2005)
DOI: 10.1007/s10886-005-5798-8

PHYTOTOXIC AND ANTIFUNGAL COMPOUNDS FROM TWO *Apiaceae* SPECIES, *Lomatium californicum* AND *Ligusticum hultenii*, RICH SOURCES OF Z-LIGUSTILIDE AND APIOL, RESPECTIVELY

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(Received December 6, 2004; accepted March 13, 2005)

Abstract—The seeds of two *Apiaceae* species, *Ligusticum hultenii* and *Lomatium californicum*, were investigated. Preliminary bioassays indicated that methylene chloride extracts of seeds of both species contained selective phytotoxic activity against monocots and antifungal activity against *Colletotrichum fragariae*. Active constituents were isolated by bioassay-guided fractionation, and the structures were elucidated by NMR and GC-MS as apiol and Z-ligustilide, isolated from *L. hultenii* and *L. californicum*, respectively. Apiol and Z-ligustilide had I_{50} values of about 80 and 600 μM, respectively, for inhibition of the growth of *Lemna paucicostata*. The methylene chloride (CH_2Cl_2) extracts of the seeds and the isolated and purified compounds were tested against the 2-methylisoborneol-producing cyanobacterium (blue-green alga) *Oscillatoria perornata*, and the green alga *Selenastrum capricornutum*. The CH_2Cl_2 extracts of both *Apiaceae* species and apiol were weakly toxic to both species of phytoplankton, while Z-ligustilide was toxic to both with a lowest complete inhibitory concentration (LCIC) of 53 μM. Seeds of *L. californicum* and *L. hultenii* were found to be rich sources of Z-ligustilide (97 mg/g of dry seed) and apiol (40 mg/g of dry seed), respectively.

Key Words V *Ligusticum hultenii*, *Lomatium californicum*, *Apiaceae*, Z-ligustilide, apiol, phytotoxic activity, antifungal activity, *Colletotrichum fragariae.*

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INTRODUCTION

The seeds of two *Apiaceae* species, *Lomatium californicum* (Torrey & A. Gray) Mathias & Constance, and *Ligusticum Hultenii* (Fernald) (Calder & Taylor), were investigated for their phytotoxic and antifungal activities. *Lomatium californicum*, also known as *California lomatium* or celery weed, is a perennial herb native to California and can be found in the southern part of Oregon on bushy slopes. *Ligusticum hultenii* is a perennial, common in the northwestern United States, particularly in Alaska. Seeds of both species have a distinct, strong aromatic odor similar to dill seeds. These two species have been previously categorized in the Umbelliferae family (renamed *Apiaceae*), but were recently reclassified in the subfamily Apioideae of the *Apiaceae* family (Watson, 2000). *Apiaceae* species possess compounds with many types of biological activities, such as apoptosis inducers, antibacterial, hepatoprotective, vasorelaxant, cyclooxygenase inhibitory, and antitumor compounds (Okuyama et al., 1991; Gonzalez et al., 1995; Liu et al., 1998; Matsuda et al., 2000; Ye et al., 2001; Pae et al., 2002). We reasoned that these species might have compounds with potential as pest management chemicals.

METHODS AND MATERIALS

Materials. Dried seeds of *L. hultenii*, and *L. californicum* were supplied by Aromagen (Albany, OR, USA). The plants were grown in Albany, OR, and the seeds were harvested in July 2002. All solvents were reagent grade and used without further purification. Extracts of the seeds were analyzed on silica gel thin-layer chromatography (TLC) plates (250 μm thickness, with fluorescent indicator; GF Uniplate Analtech, Newark, DE, USA) using 10% ethylacetate in hexane as the solvent system. Anisaldehyde spray reagent, iodine vapor, and UV light were used for the detection of compounds on the TLC plates. Column chromatography was carried out with kieselgel 60 (particle size 0.063Y0.2 mm; Merck, Germany). Seeds of chewing fescue (*Festuca rubra* L., subsp. *commutata* Gaud.), lettuce (*Lactuca sativa* L., cv. Iceberg), and creeping bent grass (*Agrostis stolonifera* L., cv. Pencross) were purchased from Turf Management, Inc., Burpee Seeds, and Tee-2-Green Corp (USA), respectively.

Instrumentation.

Extraction and Isolation of Bioactive Constituents. Dried seeds of *L. hultenii* (100 g) and *L. californicum* (100 g) were ground separately into a powder by macerating in a blender and then stirring the powdered seeds in CH_2Cl_2 (3 × 1 l each) for 1 hr at ambient temperature. The extracts were filtered through filter paper (Whatman #1), and the solvent was evaporated at 40°C to afford viscous oils (11.78 and 7.2 g, respectively).

*Isolation of Z-ligustilide (**1**).* The CH_2Cl_2 extract of *L. californicum* (5 g) was column chromatographed on silica gel (40 mm i.d., 200 mm length) using hexane and increasing amounts of CH_2Cl_2 (0Y100%). Fractions of 125 ml were collected and concentrated at 40°C, and similar fractions according to TLC profiles were combined to yield eight fractions. Each fraction was tested with TLC bioautography and phytotoxicity bioassays (see below) to identify the bioactive fractions. Fractions that possessed antifungal activity and phytotoxicity, with similar TLC profiles, were pooled and further purified by silica gel column chromatography. The identity of the active compound eluted with 50% CH_2Cl_2 in hexane was confirmed by GC-MS and comparison of and ^1H and ^{13}C NMR data with those reported in the literature (Fischer and Gijbels, 1987; Miyazawa et al., 2004) as Z-ligustilide (**1**) (2.9 g).

*Isolation of Apiol (**2**).* The CH_2Cl_2 extract of *L. hultenii* (5 g) was column chromatographed on silica gel in a similar manner as that for the isolation of Z-ligustilide to afford 10 fractions from *L. hultenii*. Each fraction was tested on TLC bioautography and phytotoxicity bioassays to identify the bioactive fractions. Fractions that possessed antifungal activity and phytotoxicity with similar TLC profiles were pooled separately and further purified by silica gel column chromatography using ethyl acetate in hexane. The active compound was eluted with 20% ethyl acetate in hexane and was identified as apiol (**2**) (1.7 g) by GC-MS and comparison of ^1H and ^{13}C NMR data with those reported in the literature (Tyagi et al., 1993).

Phytotoxicity Assays. Phytotoxicity bioassays were carried out according to Dayan et al. (2000), using chewing fescue (*Festuca rubra* L., subsp. commutata Gaud.), bentgrass (*A. stolonifera*), and lettuce (*Lactuca sativa* cv L., Iceberg), in 24-well plates. The crude extracts and fractions were tested at 1 mg/ml in 10% acetone in water.

Phytotoxicity to *Lemna paucicostata* was determined according to the method of Michel et al. (2004). Plants were grown in non-pyrogenic polystyrene sterile 6-well plates (Costar 3506, Corning Incorporated) with a lid. Each well contained 4950 µl of the Hoagland's media plus 50 µl of apiol and Z-lugustilide in acetone, with the final concentration of acetone 1%. Each well was inoculated with two, three-frond colonies of approximately the same size. Total frond area per well was recorded by the image analysis system Scanalyser (LemnaTec, Würselen, Germany) once per day from d 0 to d 7. To test the potential influence of the acetone, an acetone control was included in each experiment.

Fungicidal Assays

Pathogen Production. Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from B.J. Smith (USDA, ARS, Small Fruit Research Station, Poplarville, MS, USA). *Colletotrichum fragariae*, *C. acutatum*, and *C. gloeosporioides* were used for all pathogen and bioautography studies. The three *Colletotrichum* species were isolated from strawberry (*Fragaria* × *ananassa* Duchesne). *Botrytis cinerea* Pers.:Fr, was isolated from commercial grape (*Vitis vinifera* L.), and *Fusarium oxysporum* Schlechtend:Fr from orchid (*Cynoches* sp.) were used additionally in the microtiter assays of apiol and Z-ligustilide.

Bioautography. Bioautography on silica gel TLC plates with *Colletotrichum fragariae* was used as the preliminary bioassay to identify the antifungal activity according to a previously published method (Wedge and Nagle, 2000).

Microtiter Assay. A standardized 96-well microtiter plate assay developed for discovery of natural product fungicidal agents (Wedge and Kuhajek, 1998) was used to evaluate purified apiol and Z-ligustilide.

Algicidal Assays. Algicidal bioassays were carried out for CH_2Cl_2 extracts of *L. californicum* and *L. hultenii*, and the isolated, purified Z-ligustilide and apiol against the 2-methylisoborneol (MIB)-producing cyanobacterium *Oscillatoria perornata* and the green alga *Selenastrum capricornutum* according to previously published methods (Schrader et al., 1997) in order to determine the lowest observed effect concentration (LOEC) and the lowest complete inhibitiory concentration (LCIC).

Quantification of Active Components

Sample Preparation. For quantification, 5 g of dried seeds from each species were exhaustively extracted with CH_2Cl_2 (45 ml × 5) using a Dionex ASE 200 accelerated solvent extractor at 40°C and 68.9 bar pressure. Solvent in each sample was evaporated at 40°C under reduced pressure.

GC-MS Analysis. The concentrations of apiol and Z-ligustilide in the crude CH_2Cl_2 extracts were determined by GC-MS analysis. Standard curves were generated by linear regression, using pure apiol (R^2 = 0.98) and Z-ligustilide (R^2 = 0.99), which were isolated.

RESULTS AND DISCUSSION

The CH_2Cl_2 extracts of *L. hultenii* and *L. californicum* on lettuce (dicot) and bentgrass (monocot) were selectively phytotoxic toward bentgrass at 1.0 mg/ml with a ranking of 5 in the scale of 0Ÿ5, where 0 indicated no effect and 5

indicated no germination. The *L. hultenii* extract gave a ranking of 1 on lettuce, whereas the *L. californicum* extract gave a ranking of 2. Through bioassay-guided fractionation, the active compounds were isolated and characterized by NMR and GC-MS as Z-ligustilide (**1**) from *L. californicum* and apiol (**2**) from *L. hultenii* (Figure 1). These two compounds, (**1**) and (**2**), were further tested in a dose-response manner, in the phytotoxicity bioassays (Table 1). Both compounds were much more toxic to the two monocots, bentgrass and fescue, than to lettuce.

Phytotoxicity was further evaluated in a *L. paucicostata* bioassay (Figure 2). Apiol caused about 75% reduction of growth of *L. paucicostata* at 80 μM, whereas Z-ligustilide caused similar effects at 1000 μM. At concentrations of 166 μM and above, apiol completely inhibited the growth of *L. paucicostata*. Apiol and Z-ligustilide had I_{50} values of about 80 and 600 μM, respectively, for inhibition of the growth of *Lemna paucicostata*. At doses of apiol between 25 and 50 μM, slight stimulation of the growth of *L. paucicostata* was observed. This type of stimulation by lower doses of phytotoxins (hormesis) occurs with some other phytotoxins (Schabenberger et al., 1999). The complete inhibition of *L. paucicostata* by Z-ligustilide was observed only at 1000 μM and above. These results suggest that apiol is a more potent growth inhibitor than Z-ligustilide. Aromatic compounds having allyl or isoallyl and methylenedioxy

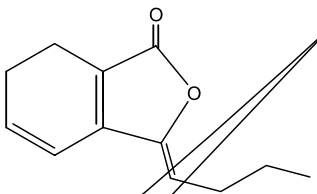

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TABLE 1. EFFECTS OF Z-LIGUSTILIDE AND APIOL ON GROWTH OF THREE HIGHER PLANT SPECIES FOR 7 D

Compound	Concentration (μM)	Lettuce	Bentgrass	Fescue
Z-ligustilide	1	0	0	0
	2	0	0	0
	10	0	1	1
	33	0	2	2
	100	0	4	3
	333	1	4	4
	1000	1	4	4
Apiol	1	0	0	0
	2	0	0	0
	10	0	1	2
	33	1	1	3
	100	1	3	4
	333	1	4	4
	1000	1	5	5

0 = no effect compared to the control.
5 = complete mortality.

groups are plant growth inhibitors (Harada et al., 1985). Myristicin, apiol, and dillapiol inhibit growth of rice, affecting mostly the foliar part of the plants without inhibiting the growth of the roots (Harada et al., 1985).

Antifungal activity was observed using bioautography with *C. fragariae* (Figure 3). In order to obtain a quantitative evaluation of antifungal activity, a microtiter plate-based bioassay was carried out according to Wedge and Kuhajek (1998). Results indicated that both apiol and Z-ligustilide had antifungal activity at the concentrations that we tested (1, 10, and 100 μM). Z-Ligustilide and apiol showed the highest activity against *B. cinerea* (Figure 4) among all the pathogens tested. Benomyl at 1 μM was more active than at 100-μM concentrations of apiol and Z-ligustilide. Low antifungal activity was observed for apiol and Z-lingustilide against *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporium* in the microtiter assay (data not shown).

The cyanobacterium *O. perornata* is a pest in commercial catfish production ponds in the southeastern region of the USA. MIB is produced by *O. perornata* and accumulates in the flesh of pond-raised channel catfish (*Ictalurus punctatus*) causing a musty "off-flavor^ that results in an unpalatable and unmarketable product. Green algae (Division Chlorophyta) such as *S. capricornutum* do not produce the off-flavor compounds commonly encountered in channel catfish aquaculture and are the preferred type of phytoplankton in catfish aquaculture ponds. Algicidal bioassay results indicated that CH_2Cl_2 extracts of both seeds had LOEC and LCIC values of 100 ppm and

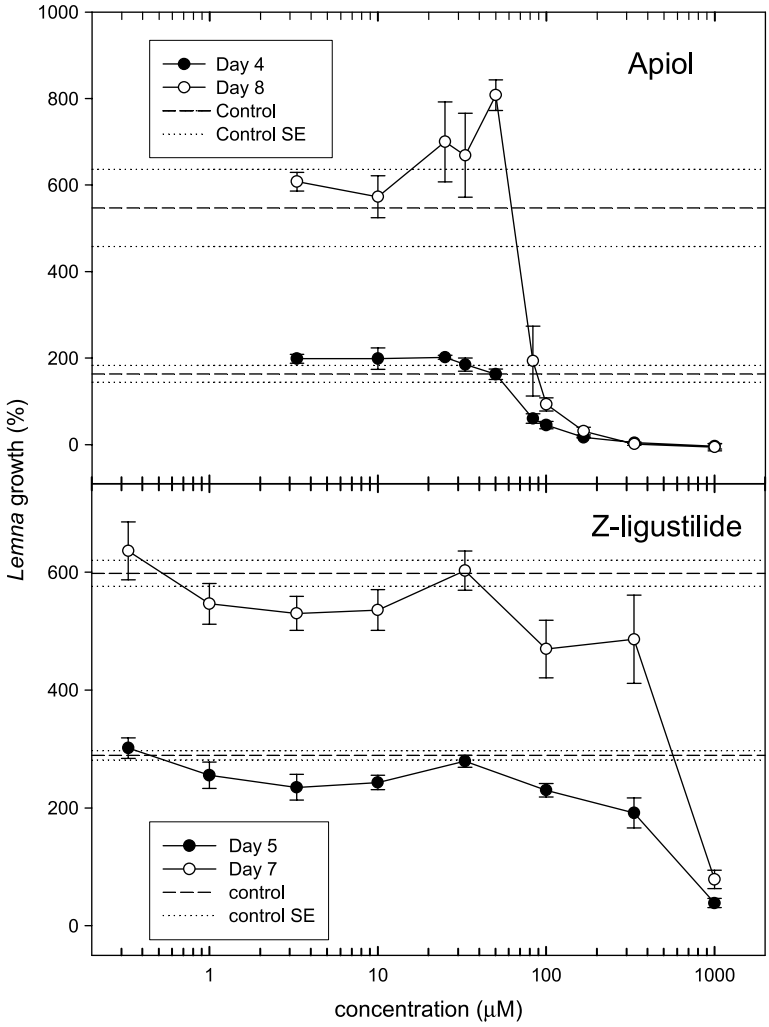

FIG. 2. Growth response of *L. paucicostata* at various concentrations of apiol and Z-ligustilide. Growth was monitored at d 4 and 8 after treatment for apiol and at d 5 and d 7 for Z-ligustilide.

higher (Table 2). Z-Ligustilide showed nonselective activity against *O. perornata* and the green alga *S. capricornutum* with LOEC and LCIC values of 53 μM (Table 2). Apiol showed no activity against either species of phytoplankton at the concentrations tested.

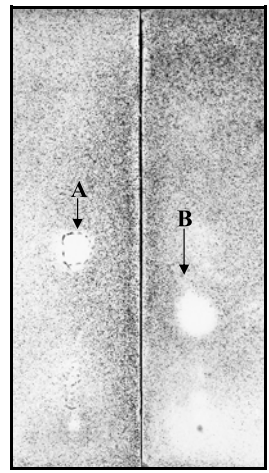

FIG. 3. Bioautography of apiol (A) and Z-ligustilide (B). The plate was sprayed with spores of *Colletotrichum fragariae*. Apiol and Z-ligustilide were chromatographed on silica gel TLC plate using 10% ethyl acetate in hexane.

Z-Ligustilide has been found previously in *Lomatium californicum* (Beauchamp et al., 1993), and is widely occurring with its *trans*-isomer in *Ligusticum* and *Lomatium* spp. (Kobayashi and Mitsuhashi, 1987; Bedrossian et al., 1998; Lu et al., 2004). Apiol can be found with ligustilides in other *Ligusticum* spp. (Brandt and Schultze, 1995).

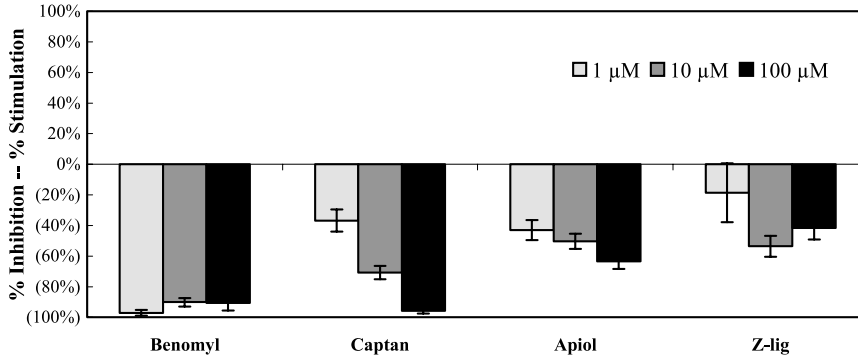

FIG. 4. Mean percent growth inhibition of *Botrytis cinerea* in response to 1, 10, and 100 μM concentrations of Z-ligustilide and apiol after 72 hr. Fungal growth showed that Z-ligustilide and apiol demonstrated less inhibition than the commercial fungicide standards benomyl and captan against *B. cinerea*.

TABLE 2. EFFECT OF CH_2CL_2 EXTRACTS OF *L. californicum* AND *L. hultenii* AND THE ISOLATED, PURIFIED Z-LIGUSTILIDE AND APIOL ON THE CYANOBACTERIUM *Oscillatoria perornata* AND *Selenastrum capricornutum*

	Test organism			
	Oscillatoria perornata		*Selenastrum capricornutum*	
Extract/compound	LOEC	LCIC	LOEC	LCIC
L. californicum	100 ppm	100 ppm	100 ppm	>100 ppm
Z-ligustilide	53 μM	53 μM	53 μM	53 μM
L. hultenii	100 ppm	>100 ppm	>100 ppm	>100 ppm
Apiol	>100 μM	>100 μM	>100 μM	>100 μM

LCIC: lowest complete inhibitory concentration.
LOEC: lowest observed effective concentration.

According to GC-MS analysis, the concentration of Z-ligustilide was 85% and that of apiol was 65% of the CH_2Cl_2 extracts of the seeds of *L. californicum* and *L. hultenii*, respectively (Figure 5). *L. californicum* and *L. hultenii* seeds were found to contain 97 mg of Z-ligustilide per g of dry seed and 40 mg of apiol per g of dry seed, respectively. These results indicated that both Z-ligustilide and apiol occur in high concentrations in these two seeds. The reason for the occurrence of these compounds in such high concentration is unclear. Insect antifeedant assays are ongoing for apiol and Z-ligustilide.

Z-Ligustilide is present in many other plants, and it is the active compound in the Chinese herbal drug *Angelica sinensis,* which is also known as Danggui or Female Gingseng (Zhao et al., 2003). This compound is chemically unstable at ambient temperature and will produce polymerized products when exposed to light and heat (Rios et al., 1998). The chemical instability is attributed by the presence of double bonds in the molecule that can undergo [2 + 2] DielsYAlder type cycloaddition, whereby one molecule serves as a diene and the other as a dienophile and resulting in insoluble complex molecules (Rios et al., 1998).

Our results show that apiol and Z-ligustilide are phytotoxic to the monocots tested in this study and are weakly antifungal. Apiol was not toxic to the MIB-producing cyanobacterium *O. perornata* and the green alga *S. capricornutum*, while Z-ligustilide was toxic, but not selective, to *O. perornata*. Z-Ligustilide is considered the active compound in the herbal drug *Angelica sinensis*, but there are no reports of the use of *L. californicum* as an herbal remedy. The possible uses of this plant or its seeds as herbal drugs as sources of Z-ligustilide are yet to be explored. Although the antimicrobial and phytotoxic activities are not sufficient for commercial use, these natural compounds could be used as templates to produce more active compounds. Furthermore, we have found Z-ligustilide to be a major compound in *L. californicum* seeds.

Acknowledgments—We thank Robert Johnson, Adriane Greer, Hope Harris, Marty Lanier, Linda Robertson, Dewayne Harries, and Ramona Pace for their valuable technical assistance. Don Stanford and Franck Wiggers are acknowledged for their assistance with GC-MS and NMR facilities.

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